PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Michael I. Watkins and Richard B. Edwards Application No.: 09/905,338 Filed: July 13, 2001 For: MULTIPLEX FLOW ASSAYS PREFERABLY WITH MAGNETIC PARTICLES AS SOLID PHASE	Examiner: Stucker Art Unit: 1648 SECOND DECLARATION UNDER 37 C.F.R. 1.131

Assistant Commissioner for Patents
Washington, D.C. 20231

MICHAEL I. WATKINS and RICHARD B. EDWARDS declare and state:

1. We are the inventors of the invention claimed in claims 21-29 and 50-58 of this Application.

2. The attached exhibit A is a photocopy of laboratory notebook entries and other materials describing experimental work that was carried out in the United States, a NAFTA country or a WTO country.

3. The experimental work described in Exhibit A was conducted prior to March 14, 1997.

4. The experimental work described in the attached Exhibit A was carried out by one or both of us, or by a person acting under the supervision of one or both of us.

5. The experimental work described in the attached Exhibit A corresponds to Examples 1 and 3 of this Application, and shows an experiment in which a plurality of types of magnetic beads was used to detect multiple analytes in a sample using flow cytometry.

6. As shown in Exhibit A, three types of beads were utilized - two sizes of SPHERO™ Carboxyl magnetic particles and one type of SINTEF™ magnetic particles. The three types of beads were differentiable from one another by particle size subrange. Each group of beads was combined with a different antigen.

7. As shown in Exhibit A and described in this patent application, the three types of beads were:

SPHERO™ Carboxyl Magnetic particles, from Spherotech, Inc., Libertyville, Illinois, USA -- poly(styrene/acrylic acid particles), 4.35 micrometers (μm) in diameter, density 1.17 g/cc, containing 12% magnetite (by weight)

SPHERO™ Carboxyl Magnetic particles, from Spherotech, Inc., Libertyville, Illinois, USA -- poly(styrene/acrylic acid particles), 3.18 μm in diameter, density 1.17 g/cc, containing 12% magnetite (by weight)

SINTEF Applied Chemistry, Trondheim, Norway -- poly(styrene/divinylbenzene) particles, 10 μm in diameter, density 1.23 g/cc, containing 17.9% magnetite/maghemite (by weight)

8. As shown in Exhibit A, pp. 1, 3 and 5, the particles were coupled to CMV, HSV2 and RUB antigens, respectively. Pages 2, 4 and 6 describe the beads. As shown in Exhibit A, pp. 7 and 8, the particles were then mixed and contacted with patient samples having known quantities of CMV, HSV2 and RUB antigens, including combinations of such

antigens, and were subjected to flow cytometry. The results are shown in the table on p. 7 of Exhibit A and below in Table II, demonstrating that multiple analytes could be detected using the magnetic particles described, in a flow cytometric immunoassay. Page 8 of Exhibit A

9. More specifically, the experimental procedure shown in the attached Exhibit A was as follows:

TABLE I
Amounts Used

Bead	Viral Antigen	Amount of Beads	Weight of Viral Antigen	Volume of Viral Antigen	Volume of Phosphate Buffer (100 mM)
4.35 μm	CMV	10 mg	225.8 μg	322.6 μL	677.4 μL
3.18 μm	HSV2	5 mg	163.0 μg	815.0 μL	185.0 μL
10 μm	RUB	5 mg	5.2 μg	104.0 μL	896.0 μL

The beads in each case were placed in test tubes and washed multiple times with 100 mM phosphate buffer, pH 6.8. The washed beads were then suspended in the volume of phosphate buffer listed in Table I, and respective antigen solution was added (CMV antigen from Chemicon International Incorporated, Temecula, California, USA; HSV2 antigen from Ross Southern Labs, Salt Lake City, Utah, USA; and RUB antigen from Viral Antigens, Memphis, Tennessee, USA) in the amount listed in Table 1. The test tubes were then rotated in end-over-end fashion overnight at room temperature. The tubes were then placed on a magnetic separator and the supernatant was drawn off and discarded. The resulting beads were washed with a wash buffer consisting of 50 mM phosphate buffer, pH 7.4, 0.01% Tween 20, 1% bovine serum albumin, 0.1% sodium azide, 150 mM sodium chloride, then again subjected to magnetic separation, and suspended in a storage buffer consisting of 50 mM phosphate buffer, pH 7.4, 5% glycerol, 1% bovine serum albumin, 0.1% sodium azide, 150 mM sodium chloride.

Procedure:

1. 100 μL each of five of patient samples (diluted 1:10 in wash buffer), of known CMV, HSV2 and RUB antibody status, were added to 12 × 75 mm polypropylene test tubes.
2. To each tube was added 100 μL of a mixture of CMV, HSV2 and RUB antigen-coated particles (described in Example 1) diluted in wash buffer.
3. The tubes were vortexed at ambient temperature for 15 minutes.
4. After vortexing, 800 μL of wash buffer was added to each tube.
5. The tubes were placed in a magnetic separator for 5 minutes and the liquid phase removed.
6. Steps 4 and 5 were repeated, but with 1000 μL of wash buffer.
7. 200 μL of a 1:300 dilution of anti-human IgG-phycoerythrin conjugate (Chemicon International Inc., Temecula, California, USA) was added.
8. The tubes were vortexed at ambient temperature for 15 minutes.
9. After this time, the samples were injected into a flow cytometer (Bryte HS, Bio-Rad Laboratories, Inc., Hercules, California, USA) equipped with a xenon arc lamp.

The results are summarized in Table II below. The data show that the positive samples had increased fluorescence relative to the negative samples. Testing of samples containing only RUB shows that essentially the same results are obtained for a particular sample whether it is assayed with only one particle size directed towards a single analyte (RUB) or with particles of different sizes, each size being directed towards a different analyte.

TABLE II

Test Results

Sample	Antibody Status			Relative Linear Fluorescence Units		
	CMV	HSV2	RUB	CMV	HSV2	RUB
CN6	+	-	+	14	7	155
CN8	+	-	+	16	6	181
CN12	-	-	+	5	7	240
CN15	-	-	+	5	6	329
23	-	+	-	5	45	43

We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the patent to which this verified states is directed.

Michael I. Watkins

Date: _____

Richard B. Edwards

Date: Dec 1, 2003

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Two Embarcadero Center, 8th Floor
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CN15	-	-	+	5	6	329
23	-	+	-	5	45	43

We further declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the patent to which this verified states is directed.

Michael I. Watkins

Michael I. Watkins

Date: 10/20/03

Richard B. Edwards

Date: _____

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Title: Adsorption of CMV Antigen to Magnetic Beads Book:

Purpose: To adsorb ~~dopper~~ Chemicon CMV antigen to magnetic beads.

Procedure

Tube	Bead	Amt Beads	Vol. Beads	Vol. CMV ($\frac{700\ \mu g}{ml}$)	Vol. 100 mM PBS
A	Spherotech 4.35 μm	10 mg	400 μl	322.6 μl	677.4 μl
B	Bangs 9803CN	2 mg	20 μl	283.0 μl	717 μl
C	Bangs 9500CN	2 mg	20 μl	199.0 μl	801 μl

1. Add appropriate beads to a labeled 12x75 mm polypropylene tube.
2. Wash bead 3x1ml with 100 mM phosphate buffer pH 6.8 by adding 1ml buffer, vortexing and placing tube in Corning magnetic separator for 3 minutes.
3. Suspend beads in the volume of 100 mM phosphate buffer indicated in above table.
4. Add the volume of CMV antigen specified in the table to the appropriate tube.
5. Place the capped tubes on an end-over-end rotator o/n @ RT.
6. The next day place the tubes on a magnetic separator for 30 minutes. Pipet off + discard supernatant.
7. Wash 4x1ml w/wash buffer by adding 1ml of wash buffer, vortexing and placing tubes in a Corning magnetic separator for 3 minutes.
8. In a similar manner wash 2x1ml w/storage buffer
9. Suspend the beads in 1ml of storage buffer and store @ 4°C.

To Page No.

Witnessed & Understood by me,	Date	Invented by M. Watkins	Date
		Recorded by	

Spherotech Inc.
1840 Industrial Dr. Suite 270
Libertyville, Illinois 60048
Tel : (708) 680 8922
Fax : (708) 680 8927

TECHNICAL DATA

PRODUCT: SPHERO™ Carboxyl Magnetic Particles, 4.0-4.5 µm
(U. S. Patent No. 5,091,206)

CAT. NO.: CM-40-10

LOT NO.: 101

SIZE: 10 ml

PARTICLE CONC.: 2.5% w/v

PRESERVATIVE: 0.05% Sodium Azide*

STORAGE: Room Temperature

CAUTION: Do not freeze.

NOTE: To achieve optimum particle suspension, resuspend by vortexing before use.

Density = 1.22 - 1.25 g/cc

% magnetite = 12%

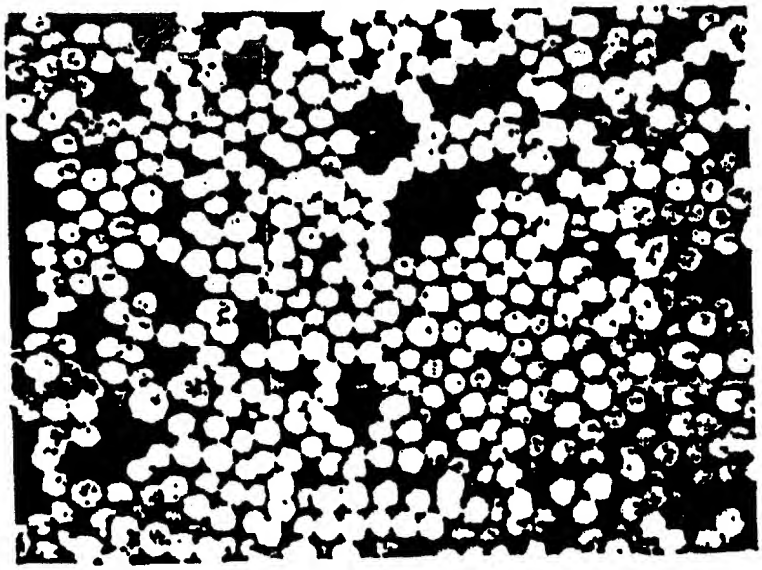
$$*\text{part./mL} = \frac{6W \times 10^{12}}{\rho \pi \phi^3}$$

w = grams/mL (soln)
ϕ = diameter (µm)
ρ = density (g/mL)

$$= \frac{(6)(0.025)(10^{12})}{(1.235)(\pi)(4.35)^3}$$

$$= 4.70 \times 10^8 \text{ part./mL}$$

SEM ANALYSIS: Magnification: 1000X. Mean Diameter: 4.35 µm



*WARNING: Sodium Azide can react with Cu and Pb in plumbing to form explosive metal azides. Flush this reagent down drains with copious amounts of water.

NOTE: FOR RESEARCH APPLICATIONS ONLY. NOT FOR DIAGNOSTIC USE.

(2)

Adsorption of HSV Antigen to 3.18 µm Spherotech beads

Purpose: To adsorb Ross Southern Labs HSV antigen to 3.18 µm magnetic beads from Spherotech.

Procedure

1. Add 200µl (5mg, 2.5%) of Spherotech 3.18 µm beads to a 12x75 mm polypropylene tube.
2. Wash beads 3x1ml with 100mM phosphate buffer pH 6.8 by adding 1ml buffer, vortexing, placing tube in Corning magnetic separator for 3 minutes and pipetting off supernatent.
3. Suspend bead in 185 µl of 100 mM phosphate buffer pH 6.8
4. Add 815µl of HSV antigen (Ross Southern Labs).
5. Cap the tube and place on a end-over-end rotator O/N @ RT.
6. The next day place the tube on a magnetic separator for 3 minutes. Pipet off & discard supernatent.
7. Wash 4x1ml w/wash buffer by adding 1ml of wash buffer, vortexing and placing tub in a Corning magnetic separator for 3 minutes.
8. In a similar manner, wash 2x1ml w/storage buffer
9. Suspend the beads in 1ml of storage buffer and store at 4°C.

Spherotech Inc.
1840 Industrial Dr. Suite 270
Libertyville, Illinois 60048
Tel : (708) 680 8922
Fax : (708) 680 8927

TECHNICAL DATA

PRODUCT: SPHERO™ Carboxyl Magnetic Particles, 3.0-3.9 µm
(U. S. Patent No. 5,091,206)

CAT. NO.: CM-30-10

LOT NO.: I01

SIZE: 10 ml

PARTICLE CONC.: 2.5% w/v

PRESERVATIVE: 0.05% Sodium Azide*

STORAGE: Room Temperature

CAUTION: Do not freeze.

NOTE: To achieve optimum particle suspension, resuspend by vortexing before use.

Density = 1.22 - 1.25 g/cc

% Magnetite = 12%

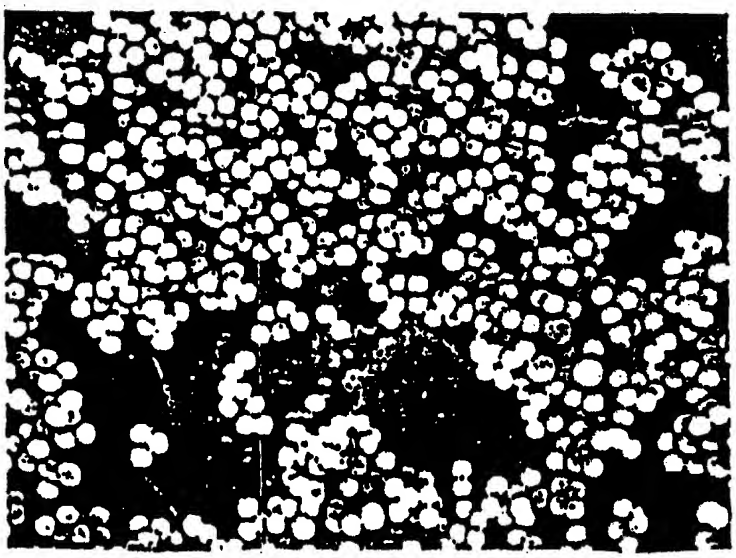
$\#part./mL = \frac{6W \times 10^{12}}{\rho \pi \phi^3}$ W = grams/mL of soln, ϕ = diameter (µm), ρ = density (g/mL)

$= \frac{(6)(0.025)(10^{12})}{(1.235)(\pi)(3.18)^3}$

$= 1.20 \times 10^9$ particles/mL

Surface Area $= \frac{6\phi}{\phi\rho} = \frac{6}{(3.18)(1.235)} = 15.3 \frac{cm^2}{mg}$

SEM ANALYSIS: Magnification: 1000X. Mean Diameter: 3.18 µm



*WARNING: Sodium Azide can react with Cu and Pb in plumbing to form explosive metal azides. Flush this reagent down drains with copious amounts of water.

NOTE: FOR RESEARCH APPLICATIONS ONLY. NOT FOR DIAGNOSTIC USE.

Project No.

Title: Adsorption of Rubella to 10μm Magnetic Sintef Beads Book No.

From Page No.

Purpose: To adsorb Rubella antigen at two different concentrations to 10μm magnetic Sintef beads.

Procedure

Tube	Vol. Rubella Antigen	Vol. 100 mM PBS pH 6.8
A	104 μL (5.2 μg)	896 μL
B	10.4 μL (0.52 μg)	989.6 μL

1. Wash 5 mg beads in tubes A & B, 3 x 1 ml of 100 mM phosphate buffer, pH 6.8 using magnetic separator (3 minutes).
2. Suspend pellet in specified volume of phosphate buffer (see table).
3. Add volume of rubella antigen specified in table.
4. Place on end-over-end rotator O/N @ RT.
5. Place on magnetic separator 3 minutes, discard supern
6. Wash 4 x 1 ml with wash buffer using 3 min. of magnetic separation.
7. Wash 2 x 1 ml with storage buffer again usi 3 min. of magnetic separation.
8. Suspend in 1 ml of storage buffer.

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Witnessed / Understood by Inc.	Date	Invented by	Date	
				5

SINTEF

SINTEF Applied Chemistry

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Enterprise No.:
NO 948 007 029 MVA

Bio-Rad Labs
4000 Alfred Nobel Drive
Hercules, CA 94547
USA

Att: Dr. Mike Watkins

Your ref.: Our ref.: Direct line: +4773592815 Trondheim,

MAGNETIC MICROSPHERES

Dear Dr. Watkins,

Please find enclosed 50mg of uncoated magnetic particles with the following specifications:

Density = 1.23 g/mL

R-509: 10µm porous, superparamagnetic particles
surface area: 89m²/g
iron content: 17.9% Fe/g particles
(in the form of magnetite Fe_3O_4 and/or maghemite γ-Fe_2O_3)
magnetic susceptibility: $12 \cdot 10^{-3}$ cgse

particles/mL =

surface area (smooth) $= \frac{6}{(10)(1.23)} = 4.88 \frac{cm^2}{mg}$

We have several types of coated particles based on these uncoated beads, where the coating both serves as pore filler (→ compact, smooth surface) and as supplier for functional groups for ligand coupling. We can also design new coatings specially for your purpose. Shortly told, we can vary the surface area and the pore sizes, the surface chemistry, the Fe-content (→ the magnetic susceptibility) and the size.

Please use always our particle number R-509 in your further correspondence concerning these particles.

We are looking forward to hear about your experiences with these magnetic beads.

Yours sincerely
SINTEF Applied Chemistry

Ruth Schmid

Ruth Schmid
Senior Research Scientist

(C

Project No.

TITLE: Flow Multi (CMV + HSV + RUB) vs single (RUB) assay Book No.

From Page No.

Purpose: To compare Eubella results in a single vs multianalyte (HSV -, CMV, RUB) format.

Observations

- controls CN 6, CN 8, CN 12, CN 15 + 23 were tested with the Gull assay and found to have the following reactivities:

	HSV	CMV	RUB
CN 6	+	−	+
CN 8	+	−	+
CN 12	−	−	+
CN 15	−	−	+
23	−	+	−

the flow results are consistant with these reactivities.

- standard 5 gave a lower signal than standard 4 in both assay formats
- controls were lower than their reported value of 134.9, 14.4, 0.5 IU/ml for Hi positive, low positive and negative controls, respectively.

To Page No.

Witnessed & Understood by me,	Date	Invented by	Date

CMV + HSV + RUB Assay

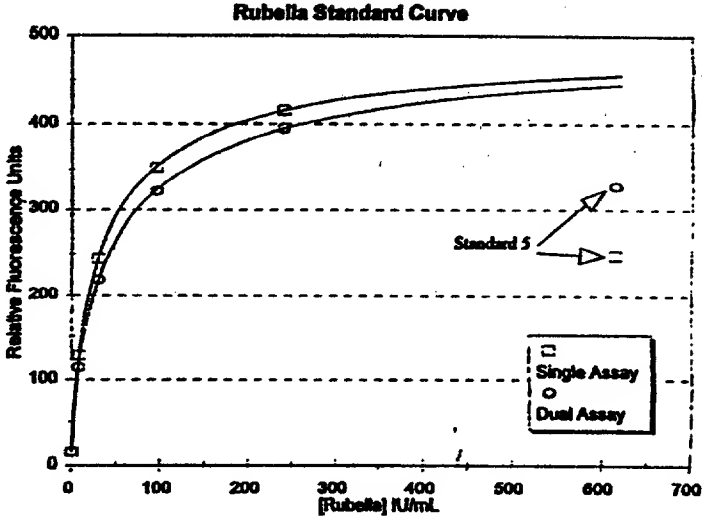
Purpose: To use compare Rubella in single versus multiassay (i.e. HSV2, CMV and RUB).

te:
erator: Watkins
s:
ads: HSV: (1/500), CMV: (1/1K)
RUB: (1/40) RUB Positive Contro.
uent: 6282-26 RUB Negative Contr
i IgG-
(B): Chemicon, AQ191E, Lot 165JDI9 (1/300) FL2 PMT: 400 (log)
mp: Xe LS1 PMT: 250 (log)
ers: G2, empty (Original Bryte) LS2 PMT: 350 (log)
annels: 2048 (log) Flowrate: 50 µL/min.

	HSV			CMV			RUB			
ample	Signal (Channels)	Rel. Linear Units	Total Counts	Signal (Channels)	Rel. Linear Units	Total Counts	Signal (Channels)	Rel. Linear Units	Total Counts	[Rub]
tUG 0							623	16	163	0.00
tUG 1							1060	129	186	7.97
tUG 2							1223	245	226	30.15
tUG 3							1304	352	115	95.41
tUG 4							1341	416	217	240.97
tUG 5							1226	248	172	31.19
ti Pos							1259	288	167	46.79
o Pos							1100	141	188	9.41
Neg							704	24	266	0.21
CN 6							1182	204	209	19.65
CN 8							1174	196	189	18.18
Cn 12							1215	236	229	27.60
Cn 15							1313	367	199	114.44
23							875	51	187	1.51
RUG 0	374	5	741	470	8	652	613	16	258	0.00
RUG 1	501	10	635	479	9	447	1054	114	208	7.88
RUG 2	633	17	514	572	13	453	1199	220	309	30.55
RUG 3	741	28	572	698	23	378	1285	323	232	94.28
RUG 4	805	37	585	765	31	412	1330	396	244	242.36
RUG 5	793	35	524	931	66	406	1289	329	253	100.88
Hi Pos	634	17	443	795	36	348	1241	265	180	49.94
Lo Pos	457	8	589	579	14	361	1063	130	211	10.11
Neg	363	5	494	700	23	442	702	24	296	0.22
CN 6	591	14	515	427	7	343	1122	155	213	14.31
CN 8	613	16	514	414	6	387	1156	181	267	19.69
Cn 12	359	5	535	425	7	365	1219	240	187	38.23
Cn 15	362	5	511	393	6	406	1289	329	178	100.88
23	346	5	516	844	45	348	838	43	246	1.21

Procedure

100 µL sample (1/10 dilution with diluent)
add 100 µL beads
↓
incubate 15 min. on vortexer @ RT
↓
Add 750 µL diluent, place on Corning magnetic separator 5 minutes
decant and let drain 1 minute on paper towels
Add 1000 µL diluent, place on Corning magnetic separator 5 minutes
decant and let drain 1 minute on paper towels
add 200 µL antihuman IgG-PE(B) - staggered additions 3 minutes apart
↓
incubate 15 min. on vortexer @ RT
↓
Read on Bryte - staggered 3 minutes apart



ALLOWED CLAIMS (U.S. Patent Application No. 09/905,338)

21. (Amended) A composition comprising a plurality of solid-phase assay reagents selectively active in a plurality of assays each for a different analyte, each said solid-phase assay reagent comprising a binding species that is selectively active in a single assay and coupled to one of a plurality of microparticles of magnetically responsive material, the sizes of said microparticles varying in size over a range that is an aggregate of a plurality of subranges, each subrange distinguishable from other subranges of said aggregate by flow cytometry and by the binding species coupled thereto, said microparticles being suitable for use in a multiplex assay procedure that includes the use of flow cytometry.

22. A composition in accordance with claim 21 in which said range is a diameter from about 0.3 micrometers to about 100 micrometers.

23. A composition in accordance with claim 21 in which said range is a diameter of from about 0.5 micrometers to about 40 micrometers.

24.. A composition in accordance with claim 21 in which the standard deviation of the particle diameters of each said subrange is less than one third of the separation of the mean diameters of adjacent subranges.

25. A composition in accordance with claim 21 in which said microparticles have a porosity substantially less than macroporous.

26. A composition in accordance with claim 21 consisting essentially of from two to 100 binding species, each selectively active in a single assay relative to the remaining binding species.

27. A composition in accordance with claim 21 in which said microparticles are comprised of a combination of a polymer and a paramagnetic substance.

28. A composition in accordance with claim 27 in which said paramagnetic substance is a metal oxide.

29. A composition in accordance with claim 27 in which said polymer is formed from monomers including carboxylate groups to permit covalent bonding of assay binding members at the microparticle surface.

50. A composition according to claim 21 in which the microparticles are comprised of from about 1% to about 75% by weight of magnetically responsive material.

51. A composition according to claim 21 in which the microparticles are comprised of from about 2% to about 50% by weight of magnetically responsive material.

52. A composition according to claim 21 in which the microparticles are comprised of from about 3% to about 25% by weight of magnetically responsive material.

53. A composition according to claim 21 in which the microparticles are comprised of from about 5% to about 15% by weight of magnetically responsive material.

54. A composition according to claim 21 in which the microparticles are differentiable by size and by a differentiation parameter other than size.

55. A composition according to claim 54 in which the differentiation parameter other than size is one or more differentiation parameters selected from the group consisting of particle composition parameters, particle physical characteristics that affect light scattering, and dyes.

56. A composition according to claim 55 in which the differentiation parameter other than size is one or more differentiation parameters selected from fluorescence, colored dyes, light scatter, light emission, and absorbance.

57. A composition according to claim 54 in which the differentiation parameter other than size is one or more fluorescence parameters.

58. A composition according to claim 57 in which two or more fluorochromes are incorporated into each subrange of microparticles.